# EVALUATION OF THREE METHODS FOR DISCRIMINATION OF *BACILLUS ANTHRACIS* FROM OTHER *BACILLUS* SPECIES.

Diane L. Dutt
Geo-Centers
Aberdeen Proving Ground, MD 21010-0068

James Rogers, Jose-Luis Sagripanti
Edgewood Chemical Biological Center
Aberdeen Proving Ground, MD 21010

## ABSTRACT

*Bacillus anthracis* shares the same ecological niche with other members of the *B. cereus* group: especially *B. cereus* and *B. thuringiensis.* Techniques that differentiate among *Bacillus* species using metabolic characteristics can be used to compliment PCR-based methods. These techniques include metabolic and fatty acid profiling. In this work we compared two metabolic-based assays and one fatty acid profiling method for their effectiveness in distinguishing *B. anthracis* from other *Bacillus spp.* Our results indicate that the methods tested vary widely in their ability to identify many *Bacillus* strains.

## INTRODUCTION

*Bacillus anthracis*, the etiological agent of anthrax, is a member of a collection of *Bacilli* known as the *Bacillus cereus* group. The *B. cereus* group includes four closely related species: *B. cereus*, *B. anthracis*, *B. thuringiensis* and *B.subtilis* var. niger (formerly identified as *B. globigii*). Distinguishing among these species is difficult (13). Indeed, Helgason *et. al*. suggest that *B. cereus*, *B. anthracis* and *B. thuringiensis* be grouped together as one species, based upon multilocus enzyme analysis (3). Alternate molecular approaches, including application of the Polymerase Chain Reaction, suggest that sequence divergence among *Bacillus* strains can be exploited for the purpose of strain identification (4-7, 9). Bacterial identification methodologies based upon traditional microbiological techniques have also been used to distinguish among *Bacillus* strains (8).

Two identification systems, Biolog and Crystal, utilize 95 and 29 biochemical tests, respectively, to accomplish this end. The incorporation of classical identification techniques into commercially available identification systems such as these is common. The well-established Biolog System has been used successfully to identify over five hundred Gram-negative (1) as well as Gram-positive and anaerobic species. The BBL Crystal Gram Positive ID Kit allows identification of 121 Gram-positive species. Excluded from the database are *B. anthracis, B. globigii* and *B. thuringiensis*.

# Report Documentation Page

*Form Approved*
*OMB No. 0704-0188*



| | | |
|---|---|---|
| 1. REPORT DATE<br>**01 JUL 2003** | 2. REPORT TYPE<br>**N/A** | 3. DATES COVERED<br>**-** |
| 4. TITLE AND SUBTITLE<br>**Evaluation Of Three Methods For Discrimination Of Bacillus Anthracis From Other Bacillus Species.** | | 5a. CONTRACT NUMBER<br>5b. GRANT NUMBER<br>5c. PROGRAM ELEMENT NUMBER |
| 6. AUTHOR(S) | | 5d. PROJECT NUMBER<br>5e. TASK NUMBER<br>5f. WORK UNIT NUMBER |
| 7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)<br>**Geo-Centers Aberdeen Proving Ground, MD 21010-0068** | | 8. PERFORMING ORGANIZATION REPORT NUMBER |
| 9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES) | | 10. SPONSOR/MONITOR'S ACRONYM(S)<br>11. SPONSOR/MONITOR'S REPORT NUMBER(S) |

12. DISTRIBUTION/AVAILABILITY STATEMENT
**Approved for public release, distribution unlimited**

13. SUPPLEMENTARY NOTES
**See also ADM001523.**

14. ABSTRACT

15. SUBJECT TERMS

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT<br>**unclassified** | b. ABSTRACT<br>**unclassified** | c. THIS PAGE<br>**unclassified** | **UU** | **7** | |

**Standard Form 298 (Rev. 8-98)**
Prescribed by ANSI Std Z39-18

An innate characteristic of the bacterial cell is the diversity of its fatty acid components. Extraction and resolution of fatty acid components by gas chromatography was developed and subsequently accepted as a method of bacterial identification (2, 10-12). In this work, we have examined the utility of fatty acid profiling for the identification of various *Bacilli*, with an emphasis placed upon members of the *B. cereus* group.

## MATERIALS AND METHODS

Avirulent bacterial cultures were purchased from the American Type Culture Collection (ATCC). Virulent *B. anthracis* strains provided to the laboratory are property of the US ARMY.

Bacterial growth media were purchased from Becton Dickenson (BD) (Sparks, MD), REMEL (Lenexa, KS) and Biolog (Hayward, CA): Trypticase Soy Agar (TSA), Blood Agar, Trypticase Soy Broth (TSB), and BUG-M medium. Chemicals were purchased from Sigma-Aldrich, Inc (St. Louis, MO). Biolog Gram-positive analysis plates, plate reader and software were purchased from Biolog, Inc. The Crystal Gram-positive test strips, test strip readers and software were purchased from Becton-Dickenson. Gas chromatographic analysis was performed using instrumentation and software purchased from Microbial ID (MIDI).

Bacterial identifications generated through use of the Crystal System were obtained in the manner described below. Bacteria obtained from a single colony were streaked onto Blood Agar plates and incubated overnight at the temperature appropriate for each strain. Cotton swabs were used to collect cells from the plate. Subsequently, the cells were suspended in GP Inoculum Fluid to a turbidity level equivalent to 0.5 McFarland Standard. The cell suspension was applied to the Analysis Panel and the sealed panel incubated at 37° C overnight. Panels were evaluated under white light and UV light using the BBL Panel viewers. Each analysis was performed in triplicate. Profile Numbers generated from each Analysis Panel were compared with the Crystal Database (BD) to produce a strain identification.

Biolog identifications were performed as follows. Cells obtained from a single colony were streaked in a cruciform pattern on BUG-M Medium. Prior to inoculation, the plates were treated with a solution of thioglycholate, in accordance with the manufacturer's directions. Once inoculated, the plates were incubated overnight at the appropriate temperature for each strain. In addition, experiments were performed in which the temperature for incubation was adjusted to either 30°C or 37°C. Following incubation, cells were collected from the margins of growth zones and suspended in GN/GP-Inoculation Fluid to a cell density of 28% ∀ 3% Turbidity. Analyses were performed in triplicate. 150 μl of cell suspension was introduced into each well of a GP-2 MicroPlate. Plates were incubated at 37°C overnight and analyzed using a Biolog MicroStation Reader and Dangerous Pathogens Database (Biolog).

Fatty acid analysis of bacterial strains was accomplished using the protocol outlined by Microbial ID. The protocol involved the following steps. Bacterial cultures were prepared by streaking cells obtained from a single colony in a quadrant pattern onto Blood Agar. 40 mg of cells were harvested from the third quadrant, suspended in 1 ml Reagent 1 (0.169M NaOH:Methanol, 1:1) and incubated at 100°C for 30 min. 2 ml of Reagent 2 (6N HCl:Methanol, 13:11) was added and the sample incubated at 80°C for 10 min after which 1.25 ml of Reagent 3 ( Hexane:Methyl tert-butyl ether, 1:1) was added. The sample was mixed for 10 min using a rotator. The aqueous phase was removed and the solution washed with 3 ml of Reagent 4 (0.3M NaOH) for 10 min. The organic phase was analyzed using an Agilent

Technologies 6850 Series Gas Chromatograph. Chromatographic profiles were compared to those present in the Clin40, TSBA and Bioterrorism Databases (MIDI).

## RESULTS

### IDENTIFICATION OF BACTERIAL STRAINS USING THE CRYSTAL SYSTEM

Repeated analysis of the control strain, *S. pyogenes*, indicated that the Crystal System was being manipulated properly for the identification of the control and test strains. At the onset of this work, we recognized that the *Bacillus spp.* represented in the Crystal System database was excluded a number of strains that we proposed to test. The strains absent from the database are *B. mycoides*, *B. thuringiensis* and *B. anthracis*. Our analysis of various *Bacllius* strains

Table 1. Bacterial Identification – Crystal System

| **Species Tested** | **Identification** | **Correct Identification** |
|---|---|---|
| *Streptococcus pyogenes* | *S. pyogenes* | 3/3 |
| *Bacillus cereus* | *B. cereus* | 4/5 |
| *Bacillus anthracis* Δ Stern | Unidentified | 0/5 |
| *Bacillus anthracis* Pasteur | Unidentified | 0/5 |
| *Bacillus licheniformis* | *B. licheniformis* | 2/2 |
| *Bacillus mycoides* | Unidentified | 0/5 |
| *Bacillus pumilus* | *B. pumilus* | 2/6 |
| *Bacillus thuringiensis* | *Bacillus licheniformis* | 0/5 |
| *Bacillus sphaericus* | *B. sphaericus* | 2/2 |

produced correct identifications less consistently than the control strain. *B. thuringiensis* was identified in every instance as *B. licheniformis,* while *B. licheniformis* was correctly identified. *B. cereus* was identified correctly 80% of the time and *B. sphaericus*, 100% (Table 1). Reliable identification of *B. pumilus* was not observed. Identification codes produced by Crystal Identification Panels for *B. mycoides* and *B. anthracis* were not recognized by the database, precluding identification.

Repeated analysis of *B. anthracis* strains resulted in the generation of reliable identification codes for two *B. anthracis* strains, Δ Sterne and Pasteur, as well as *B. mycoides* (Table 2).

Table 2. Identification Codes for *Bacillus spp.*

| **Species Tested** | A | B | **C** | **D** | **E** | **F** | **G** | **H** | **I** | **J** | **n** |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *S. pyogenes* | 1 | 7 | 6 | 5 | 7 | 7 | 7 | 5 | 6 | 1 | 3 |
| *B. anthracis* Δ Sterne | 0 or 1 | 7 | 6 | 5 | 4 | 5 | 2 or 3 | 1 | 6 | 3 | 5 |
| *B. anthracis* Pasteur | 1 | 7 | 6 | 5 | 4 | 4 or 5 | 2 or 3 | 7 | 6 | 3 | 5 |
| *B. mycoides* | 2 | 5 | 3 | 4 | 5 | 7 | 7 | 5 | 5 | 3 | 5 |

## IDENTIFICATION OF BACTERIAL STRAINS USING THE BIOLOG SYSTEM

Bacterial identification of *B. anthracis* strains using the GP-Microplates , in general, identified test strains correctly. *B. anthracis* Zim 89 was identified correctly in one third of the trials involving that strain (Table 3). The alternative to a correct identification for *B. anthracis* Zim 98 was that the strain assayed as "No Match", indicating that the identification code had no matching pattern in the Microlog Database. All remaining *B. anthracis* strains tested were identified correctly.

Table 3. Bacterial Identification – Biolog System

| ***B. anthracis*** **Strains Tested** | **Dangerous Pathogens Database** | **Correct Identification** |
|---|---|---|
| *B. anthracis* Nebraska | *B. anthracis* | 3/3 |
| *B. anthracis* ZIM 89 | *B. anthracis* | 1/3 |
| *B. anthracis* LA1 | *B. anthracis* | 3/3 |
| *B. anthracis* 1090 | *B. anthracis* | 5/5 |
| *B. anthracis* G28 | *B. anthracis* | 3/3 |
| *B. anthracis* CDC 684 | *B. anthracis* | 5/5 |

## IDENTIFICATION OF BACTERIAL STRAINS USING THE MICROBIAL I. D. SYSTEM

Identification of bacterial strains based upon unique fatty acid profiles consistently produced correct identifications for both control strains (*P. aeruginosa, Steno. maltophelia*) while typically identifying *B. anthracis* strains correctly when using either the Clin 40 or Bioterrorism Databases. *B. anthracis* is not a component of the TSBA database and therefore the possibility of incorrect identification by the TSBA Database is excluded (Table 4).

Virulent *B. anthracis* strains were used to test the utility of the MIDI system for bacterial identification in the Biological Safety Level-3 (BSL-3) containment laboratory. Using the Bioterrorism Database, the control strains and *B. anthracis* strains were identified correctly (Table 5).

Table 4. Analysis of Avirulent *Bacillus* Strains – MIDI

| ***Bacillus* strains** | **Clin 40 Database** | **TSBA Database** | **Bioterrorism Database** | **(n)** |
|---|---|---|---|---|
| *B. anthracis* Pasteur | *B. anthracis* | no match | *B. anthracis* | 8/8 |
| *B. anthracis* )Sterne | *B. anthracis* | no match | *B. anthracis* | 8/8 |
| *B. cereus* | *B. cereus* | *B. cereus* | no match | 4/4 |
| *B. globigii* SB512 | *B. subtilis* | *B. subtilis* | no match | 4/4 |
| *B. licheniformis* 12759 | *B. licheniformis* | *B. licheniformis* | no match | 4/4 |
| *B. megaterium* | *B. megaterium* | *B. megaterium* | no match | 4/4 |
| *B. mycoides* | not tested | *B. mycoides* | not tested | 4/4 |
| *B. pumilus* | not tested | *B. pumilus* | no match | 4/4 |
| *B. subtilis* | *B. subtilis* | *B. subtilis* | no match | 8/8 |
| *B. thuringiensis* | *B. cereus* | not tested | no match | 4/4 |

## CONCLUSIONS

Identification of members of the genus *Bacillus* was accomplished using the three methodologies employed. We found the Crystal System to be limited in its utility by the paucity of *Bacillus* species represented in the database. Note that an expansion of the database can be achieved through repetitive examination of known bacterial strains. Reproducibility of species identification codes indicates that the user, through the incorporation of user-generated Identification Codes, could expand the database. Indeed, through this work, reproducible Identification Codes for *B. anthracis Pasteur, B. anthracis* ) Sterne and *B. mycoides* were generated.

The Biolog System was demonstrated to be reliable for the identification of virulent strains of *Bacillus anthracis*, using the Dangerous Pathogens Database.

Fatty acid profiling using the Microbial ID gas chromatography system provided correct bacterial identifications for twenty-nine of the thirty-one strains tested. Distinguishing among members of the *B. cereus* group presented the greatest challenge. The identification of *B. globigii* as *B. subtilis* may be attributed to a change in nomenclature. Currently, the classification of *B. globigii* is that of *B. subtilis* var. niger. Fatty Acid profile comparisons using the Clin 40 Database indicated that, *B. thuringiensis* could not be distinguished from the closely allied *B. cereus*. Queries of the Bioterrorism database regarding all non-*B. anthracis* strains reported no false positive results. Obversely, all queries of the Bioterrorism Database regarding *B. anthracis* strains resulted in correct identification of all strains considered.

In this work, we compared the effectiveness of three methodologies for the identification of *Bacillus spp*. with special attention to the Select Agent *Bacillus anthracis*. Although the Crystal and Biolog identification systems identified successfully many species of *Bacilli*, overall, the MIDI System produced correct identifications for *B. anthracis* strains, reported no false positives, false negatives and required a minimal analysis time.

Table 5. Analysis of Virulent *Bacillus* Strains - MIDI

| **Strain** | **Identification** | **Correct Identification** |
|---|---|---|
| *Pseudomonas aeruginosa* | *P. aeruginosa* | 9/9 |
| *Stenotrophomonas maltophelia* | *S. maltophelia* | 9/9 |
| *B. anthracis* 1024 Albia | *B. anthracis* | 7/9 |
| *B. anthracis* 1024 SK128 | *B. anthracis* | 6/6 |
| *B. anthracis* 1032M | *B. anthracis* | 6/6 |
| *B. anthracis* 1087 Scotland | *B. anthracis* | 6/6 |
| *B. anthracis* 1090 | *B. anthracis* | 6/6 |
| *B. anthracis* 1928 | *B. anthracis* | 9/9 |
| *B. anthracis* 5444 St. Mary's | *B. anthracis* | 6/6 |
| *B. anthracis* CDC 471 | *B. anthracis* | 6/6 |
| *B. anthracis* CDC 684 | *B. anthracis* | 6/6 |
| *B. anthracis* Colorado | *B. anthracis* | 6/6 |
| *B. anthracis* EB1 | *B. anthracis* | 6/6 |
| *B. anthracis* FLA U770 | *B. anthracis* | 6/6 |
| *B. anthracis* G28 | *B. anthracis* | 5/6 |
| *B. anthracis* LA1 | *B. anthracis* | 6/6 |
| *B. anthracis* NCTC 109 Paddington IV | *B. anthracis* | 6/6 |
| *B. anthracis* NCTC 7752 | *B. anthracis* | 6/6 |
| *B. anthracis* Nebraska | *B. anthracis* | 6/6 |
| *B. anthracis* SK102 | *B. anthracis* | 6/6 |
| *B. anthracis* ZIM 89 | *B. anthracis* | 6/6 |

## REFERENCES


1. Bochner, B. R. 1991. Identification of over 500 Gram-negative species by a single test panel. Am. Clin. Lab. 10:157-158.
2. Buyer, J. S. 2002. Rapid sample processing and fast gas chromatography for identification of bacteria by fatty acid analysis J. Microbiol. Meth. 51:209-15.
3. Helgason, E., O. A. Okstad, D. A. Caugant, H. A. Johansen, A. Fouet, M. Mock, I. Hegna, and Kolsto 2000. *Bacillus anthracis, Bacillus cereus*, and *Bacillus thuringiensis* - one species on the basis of genetic evidence Appl. Envir. Microbiol. 66:2627-2630.
4. Jackson, P. J., K. K. Hill, M. T. Laker, L. O. Ticknor, and P. Keim 1999. Genetic comparison of Bacillus anthracis and its close relatives using amplified fragment length polymorphism and polymerase chain reaction analysis J. Appl. Microbiol. 87:263-269.
5. Keim, P., A. Kalif, J. Schupp, K. Hill, S. E. Travis, K. Richmond, D. M. Adair, M. Hugh-Jones, C. R. Kuske, and P. J. Jackson 1997. Molecular evolution and diversity in *Bacillus anthracis* as detected by amplified fragment length polymorphism markers J. Bacteriol. 179:818-824.

6. Keim, P., A. M. Klevytska, L. B. Price, J. M. Schupp, G. Zinser, K. L. Smith, M. E. Hugh-Jones, R. Okinaka, K. K. Hill, and P. J. Jackson 1999. Molecular diversity in Bacillus anthracis J. Appl. Microbiol. 87:215-217.
7. Keim, P., L. B. Price, A. M. Klevytska, K. L. Smith, J. M. Schupp, R. Okinaka, P. J. Jackson, and M. E. Hugh-Jones 2000. Multiple-locus variable-number tandem repeat analysis reveals genetic relationships within *Bacillus anthracis* J. Bacteriol. 182:2928-2936.
8. Kournikakis, B., C. Bateman, and J. W. Cherwonogrodzsky 2000. Characterization of 21 strains of *Bacillus anthracis*. Technical Memorandum. National Defense.
9. Lee, M. A., G. Brightwell, D. Leslie, H. Bird, and A. Hamilton 1999. Fluorescent detection techniques for real-time multiplex strand specific detection of *Bacillus anthracis* using rapid PCR J. Appl. Microbiol. 87:218-223.
10. Lin, S., H. Schraft, J. A. Odumeru, and M. W. Griffiths 1998. Identification of contamination sources of *Bacillus cereus* in pasterurized milk J. Food Microbiol. 43:159-71.
11. Moore, L. V., D. M. Bourne, and W. E. Moore 1994. Comparaitve distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic gram-negative bacilli Int. J. Sys. Bacteriol. 44:338-47.
12. Tang, Y. W., N. M. Ellis, M. K. Hopkins, D. H. Smith, D. E. Dodge, and D. H. Persing 1998. Comparison of phenotypic and genoptypic techniques for identification of unusual aerobic pathogenic gram-negative bacilli J. Clin. Microbiol. 36:3574-9.
13. Turnbull, P. C. B. 1999. Definitive identification of *Bacillus anthracis* - a review J, Appl. Microbiol. 87:237-240.